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Thin-Layer (Planar) Chromatography

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Sterols are steroid compounds widely distributed in various biological materials, e.g. variety plant and animal lipids, medications, food and dietary supplements. They are basic metabolites in living organisms and they are also precursors of a variety of bile acids, provitamins and steroid hormones. Therefore the analysis of sterols is important in biochemistry, medicine and pharmacy. There is considerable interest in the study of the relationship of plasma cholesterol concentrations to the risk of developing coronary artery disease. Determination of phytosterols and cholesterol is important for the diagnosis of phytosterolaemia and in dietary treatment of hypercholesterolaemia.

The collective name for sterols has been adopted for all naturally occurring crystalline unsaponifiable steroid alcohols. The basic sterol is 5α-cholestane, and the structure numbering system for sterols are given in **Figure 1**. In general, these compounds are 3-monohydroxysteroids, having 27, 28 or 29 carbon atoms and nearly all have one or more double bonds. The double bond is most commonly found at position 5, with double bonds at C_7 and C_{22} also being prevalent. Sterols are classified into five groups: cholesterol and its companions, zoosterols, phytosterols, mycosterols and vitamin D. Examples of sterols from each group are given in **Table 1**.

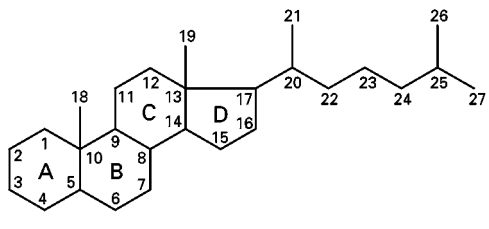

Figure 1 5α-Cholestane skeleton and numbering system for sterols.

There are four principal methodologies used in sterol chromatography: gravity flow column liquid chromatography (GCC), high performance liquid chromatography (HPLC), gas chromatography (GC) and thin-layer chromatography (TLC). For its selectivity, sensitivity and efficiency, TLC is one of the most frequently employed procedures for the separation of sterols, both for their characterization and for their quantitative analysis.

Preparation of Sample

Since sterols are present in different materials, sample preparation is a very important part of their analysis.

The first step is an extraction procedure which is performed either directly on the previously deproteinized sample or after cleavage of any conjugates present. Diethyl ether, dichloromethane, ethyl acetate, chloroform and other medium polarity organic solvents can be used for extraction.

The next step is purification of the extract or, more exactly, separation of sterols from other lipids, usually by TLC on a silica gel G plate using an *n*-heptane–diethyl ether–acetic acid (85 : 15 : 1) mixture as the mobile phase. Under these conditions the cholesterol and phytosterols are concentrated in one band. Separation and quantitative analysis of individual sterols are performed after elution from the plate by GC, TLC or some other technique.

Some sterols (e.g. vitamin D) are sensitive to atmospheric oxygen, traces of acids and bases, light and heat. Therefore, all steps should be carried out in a cool place, protected from exposure to direct light, and only highly purified solvents should be used.

Stationary Phases and Solvent Systems

Generally, the chromatographic separation of individual sterols is difficult due to the large number of epimers and unsaturated isomers. Various forms of silica gel are most frequently used in the TLC of

Table 1 Examples of sterols

Number of carbon atoms	*Trivial name*	*Systematic name*	*Group of sterols*[a]
27	Vitamin D_3 (cholecalciferol)	$\Delta^{5,7}$-Cholestadiene-3β-ol with open B-ring	5
	Cholesterol	Δ^{5}-Cholestene-3β-ol	1
	7-Dehydrocholesterol (provitamin D_3)	$\Delta^{5,7}$-Cholestadiene-3β-ol	1
	Desmosterol	$\Delta^{5,24}$-Cholestadiene-3β-ol	2
	Dihydrocholesterol	5α-Cholestane-3β-ol	1
28	Brassicasterol	24-β-Methyl-$\Delta^{5,22}$-cholestadiene-3β-ol	3
	Campesterol	24-α-Methyl-Δ^{5}-cholestene-3β-ol	3
	Ergosterol (provitamin D_2)	24-β-Methyl-$\Delta^{5,7,22}$-cholestatriene-3β-ol	3
	Fungisterol	24-β-Methyl-Δ^{7}-5α-cholestene-3β-ol	4
	Vitamin D_2 (ergocalciferol)	24-β-Methyl-$\Delta^{5,7,22}$-cholestatriene-3β-ol, with open B-ring	5
29	Dihydro-β-sitosterol	24-β-Ethyl-5α-cholestane-3β-ol	3
	β-Sitosterol	24-β-Ethyl-Δ^{5}-cholestene-3β-ol	3
	Stigmasterol	24-β-Ethyl-$\Delta^{5,22}$-cholestadiene-3β-ol	3
30	Lanosterol	4,4,14-Trimethyl-$\Delta^{8,24}$-cholestadiene-3β-ol	1

[a]Group of sterols: 1, cholesterol and its companions; 2, zoosterols; 3, phytosterols; 4, mycosterols; 5, vitamin D.

sterols; separation can be classified, according to the type of stationary phase, into four main groups: silica gel, silver nitrate silica gel, reversed-phase and bromine-system TLC.

Silica Gel TLC

The separation of individual sterols by adsorption chromatography on silica gel pre-coated plates is relatively easy when there are differences in the type, number, position or configuration of polar groups, but it is difficult in the absence of such differences.

The chromatographic properties of sterols on silica gel G plates using two solvent systems, benzene–diethyl ether (9 : 1) and benzene–diethyl ether (85 : 15) have been studied. The resulting R_F values are listed in **Table 2**. However, the separation of sterols with these systems is incomplete.

Table 2 Relative retentions of some sterols on silica gel G

Sterol	R_F *value*	
	System I	*System II*
Cholesterol	0.20	0.47
7-Dehydrocholesterol	0.19	
Desmosterol	0.20	0.46
Brassicasterol	0.20	
Ergosterol	0.19	0.43
Vitamin D_2	0.19	
β-Sitosterol	0.20	
Stigmasterol	0.20	
Lanosterol	0.31	0.62

Stationary phase: silica gel G; mobile phase: (I) benzene–diethyl ether (9 : 1); (II) benzene–diethyl ether (85 : 15); visualization: sulfuric acid.
Date from Xu *et al.* (1988).

The separation of cholesterol and cholesterol esters from other lipid fractions on silica gel pre-coated plates using the following solvent systems: first, chloroform–methanol–water (65 : 25 : 4), second, *n*-hexane–acetone (100 : 1) and third, *n*-hexane–acetone (100 : 3) has also been reported. The plates are developed with the first solvent system to 8 cm from the origin. After drying, the plates are developed to 18 cm above the origin with either of the other solvent systems. Cholesterol and four cholesterol ester subfractions are separated from other lipid fractions.

Silica gel GF plates have been used to separate cholesterol, cholesteryl propionate and low molecular weight cholesteryl esters by one-stage one-dimensional TLC. This work employed four solvent systems, the best separation among cholesteryl formate, cholesteryl acetate and cholesteryl propionate was achieved using chloroform–diethyl ether–acetic acid–1-propionic acid (92 : 1.5 : 1 : 5 : 0.5).

The solvent systems used for the separation of eight 3β-sterols of considerable biological interest, which differ only in ring B and/or in the side chain, on silica gel G pre-coated plates has also been investigated. The separation was performed using first, cyclohexane–ethyl acetate–water (600 : 400 : 1); second, cyclohexane–heptane (1 : 1), third, cyclohexane–ethyl acetate–water (1560 : 440 : 1), and fourth, isooctane–carbon tetrachloride (19 : 1). Differences in resolving power between polar and nonpolar systems were observed. Resolution of the pairs with saturated and unsaturated side chain β-sitosterol–stigmasterol and cholesterol–desmosterol was finally effected by a mixture of saturated hydrocarbons.

Separation of vitamin D from its close structural analogues, including provitamin D, irradiation

products of provitamin D and decomposition products, has been carried out on nonimpregnated layers of silica gel G with the solvent system cyclohexane–dichloroethane–diethyl ether (5 : 3 : 2). TLC has also been used for pre-purification of saponified samples before GC analysis as well as for their *in situ* quantitative analysis. Determination of the maximum permissible limit of concentration of ergosterol in ergocalciferol using silica gel G as the stationary phase with a cyclohexane–peroxide-free ether (1 : 1) mixture containing 0.1 g L^{-1} butylhydroxytoluene is an official method in the European Pharmacopoeia 1997.

In general, monosaturated sterols like cholesterol, provitamin D (e.g. ergosterol) and vitamins D are separable, but closely related sterols like cholesterol, stigmasterol and β-sitosterol are not resolved on silica gel.

Silver Nitrate TLC (Argentation Chromatography)

Several methods have been published for separation of structurally related sterols. A procedure utilizing π-complex formation between Ag(I) ions and the double bonds occurring in various locations in the sterol molecules has been frequently applied. Argentation TLC is a method for separating compounds based on differences in number and position of double bonds in the molecule. In this case, silica gel is suspended in an aqueous solution of silver nitrate before spreading on the plate. Silver nitrate can also be sprayed on to a pre-prepared layer.

Argentation TLC of sterols has been thoroughly investigated. The R_S values (relative retention relating to cholesterol) of selected sterols and sterol acetates separated on silica gel G–silver nitrate pre-coated plates using the solvent systems chloroform–diethyl ether–acetic acid (97 : 2.3 : 0.5) and chloroform–light petroleum (b.p. 60–80°C)–acetic acid (25 : 75 : 0.5) are listed in **Table 3**.

Sterols that differ in the number and position of double bonds are clearly separated by means of silver nitrate TLC, but separation of cholesterol from the phytosterols was not achieved.

Reversed-phase TLC

One of the pioneer works on reversed-phase TLC (RP-TLC) used silica gel impregnated with paraffin oil as the stationary phase and methyl ethyl ketone as the mobile phase for the separation of lipids, including cholesterol esters. Kieselguhr G has been used following impregnation with undecane as the stationary phase with the solvent systems acetic acid–water (90 : 10) and acetic acid–water (92 : 8) for the separation of sterols and sterol acetates, respectively. R_S values of some sterols and sterol acetates obtained by RP-TLC are given in **Table 4**.

Table 3 Separation of sterols and sterol acetates on silica gel G–silver nitrate layers

Sterol	*R_S value*	
	System I (sterols)	*System II (sterol acetates)*
Cholesterol	≡ 1.00	≡ 1.00
7-Dehydrocholesterol	0.44	0.43[a]
Dihydrocholesterol	1.14	1.25
Brassicasterol	0.98	0.68
Vitamin D_2	0.64	
Dihydro-β-sitosterol	1.14	1.30
β-Sitosterol	1.00	1.00
Stigmasterol	0.98	0.87
Lanosterol	1.70	0.78

[a]After two developments.
Stationary phase: silica gel G–silver nitrate. Mobile phase: (I) chloroform–diethyl ether–acetic acid (97 : 2.3 : 0.5); (II) chloroform–light petroleum (b.p. 60–80°C)–acetic acid (25 : 75 : 0.5). Visualization: dibromofluorescein.
Data from Copius-Peereboom and Beekes (1965).

The critical pair cholesterol–brassicasterol was not separated in these RP systems and RP-TLC separation according to the degree of unsaturation using the so-called bromine system was suggested (see below).

A good separation of the pairs vitamin D_2/D_3 and pre-vitamin D_2/D_3 on silica gel and Kieselguhr G impregnated with silicone oil eluted with acetone–water mixture has been achieved.

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The Bromine System TLC

The separation of unsaturated sterols from their saturated analogues can be substantially improved by

Table 4 R_S values of some sterols and sterol acetates obtained in RP-TLC

Sterol	*R_S value*	
	System I (sterols)	*System II (sterol acetates)*
Cholesterol	≡ 1.00	≡ 1.00
7-Dehydrocholesterol	1.12	1.26
Dihydrocholesterol	0.90	0.89
Brassicasterol	1.00	1.00
β-Sitosterol	0.86	0.83
Stigmasterol	0.93	0.91
Lanosterol	0.84	0.97

Stationary phase: Kieselguhr G impregnated with undecane; mobile phase: (I) acetic acid–water (90 : 10); (II) acetic acid–water (92 : 8), visualization: phosphomolybdic acid.
Data from Copius-Peereboom JW and Beekes HW (1962). The analysis of mixtures of animal and vegetable fats. III. Separation of some sterols and sterol acetates by thin-layer chromatography. *Journal of Chromatography* 9: 316.

Table 5 R_S values of some sterols acetates in the bromine system TLC

Sterol acetates	R_S
Cholesterol	≡ 1.00
7-Dehydrocholesterol	Front
Dihydrocholesterol	0.85
Brassicasterol	1.13
β-Sitosterol	0.82
Stigmasterol	1.06
Lanosterol	Front

Stationary phase: Kieselguhr G impregnated with undecane; mobile phase: acetic acid–acetonitrile (1 : 3) + 0.5% of bromine, visualization: antimony trichloride.
Data from Copius-Peereboom and Beekes (1965).

bromination. After spotting the sterol sample at the starting zone, some drops of bromine are spotted on the same place. The plate is then developed with a benzene–ethyl acetate mixture (2 : 1), by means of which the spots of cholesterol and dihydrocholesterol are separated. Thus, sterol acetates have been separated on Kieselguhr G impregnated with undecane using solvent system acetic acid–acetonitrile (1 : 3) + 0.5% bromine. R_S values of some sterol acetates are given in **Table 5**.

Bromination of the double bonds before or during chromatography completely changes the mobilities of the unsaturated compounds, promoting their separation from the saturated derivatives. In this way the critical pair cholesterol–brassicasterol can be clearly separated.

Detection and Quantitation

Detection

Sterols that have UV absorbance can be detected at 254 nm (providing that TLC separation is performed on a layer with a fluorescent indicator). Since a number of sterols do not have UV absorbance suitable for detection, most applications still involve visualization based on chemical reactions. Visualization procedures used to detect and characterize sterols are well

Table 6 Detection procedures based on chemical reactions used in TLC analysis of sterols

Detection reagent	*Visualization procedure*	*Sterols*
Acids		
Perchloric acid (70%)	Spray	Vitamin D_2
Phosphomolybdic acid (15% ethanolic solution)	Spray, heat at 110°C for 10 min	Various sterols
Sulfuric acid (conc. or 50%)	Spray, heat at 110°C for 15 min, observe in day and UV light before and after heating	C_{27} sterols, cholestane and lanostane series, ergosterol
Metal salts		
Antimony pentachloride (30% in chloroform)	Spray, heat at 120°C for 5 min	Various sterols
Antimony trichloride (50% in conc. acetic acid)	Spray, heat at 100°C for 10 min	Brominated sterols
Cadmium chloride (50% in 50% ethanol)	Spray, heat at 90°C for 15 min, observe in UV light	Brominated sterols, cholesteryl esters
Copper sulfate		Saturated species
Cupric acetate (3% in 8% phosphoric acid)	Spray, heat at 150°C for 30 min	Unsaturated species
Aldehydes		
p-Ansialdehyde (1% in acetic acid–sulfuric acid (98 : 2) mixture)	Spray, heat at 90°C for 10 min	Sterol and sterol acetates
Salicylaldehyde	Spray with pure salicyladehyde, heat at 80°C for 5 min, spray with 0.5 mol L^{-1} sulfuric acid, heat again at 90°C for 10 min	Sterol and sterol acetates
Vanillin (0.5% in sulfuric acid–ethanol (4 : 1) mixture)	Spray, heat at 100°C for 5 min	Cholestanols and cholestanones
Ketone reagents		
2,4-Dinitrophenylhydrazine (5% in methanol)	Spray and spray again with conc. sulfuric acid	Ketonic sterols

established. Detection reagents can be classified into four groups: acids, metal salts, aldehydes and ketone reagents. The most frequently used detection procedures are listed in **Table 6**.

TLC coupled with flame ionization detection (TLC-FID) has been used to detect sterols. The separation is performed on specially prepared thin quartz rods coated with adsorbent sintered on to the surface of thin rods. The adsorbent is usually silica gel and the solvent system is basically the same as in classical TLC.

TLC-FID is a useful technique for the separation of cholesterol and its esters from other lipid classes. Separation of individual sterols, especially phytosterols (e.g. β-sitosterol, campesterol, brassicasterol, stigmasterol, etc.) using TLC-FID is not possible. On the other hand, groups of sterols differing in the number of methyl groups in position 4 (i.e. 4-demethylsterols, 4-methylsterols and 4,4-dimethylsterols) can easily be separated by TLC-FID.

The TLC separation of sterols is often used for preparative purposes. After elution from the plate, the sterols can be analysed by some other technique (spectrophotometry, fluorimetry, GC, GC-MS, HPLC), which is why it is sometimes necessary to visualize them using nondestructive reagents such as iodine vapour, water spray or fluorescent reagents (e.g. fluorescein, Rhodamine B). Fluorescent reagents can be incorporated in the slurry, during the preparation of the layer. An example of a nondestructive detection using radiolabelled [4-^{14}C] cholesterol and cholesteryl [^{14}C]oleate added as internal standards has also been reported where desmosterol in monkey spermatozoa was separated on silica gel G. The free sterol band containing both cholesterol and desmosterol was detected, extracted from the plate and after, derivatization, analysed by GC.

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Quantitative *in situ* Analysis

Due to progress in plate technology and instrumentation, modern TLC has become a comparable method to other chromatographic techniques in terms of accuracy, precision, reliability and repeatability. In modern TLC, the main steps are automated, including the sample application on the plate – the step considered the most critical for quantitative evaluation. Several examples of direct quantitation of sterols on TLC plate are discussed below.

Cholesterol ester mapping of human serum by high performance TLC (HPTLC) has been performed. Quantitative analysis was carried out at 546 nm after postchromatographic derivatization with phosphomolybdic acid. The densitogram of a standard mixture containing cholesterol esters is given in **Figure 2**.

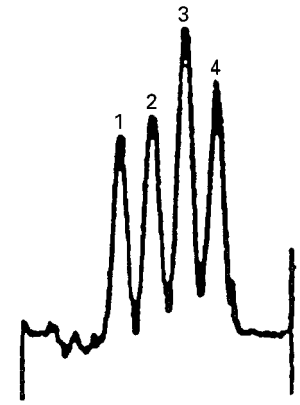

Figure 2 Densitogram of a standard mixture containing 33 ng of each cholesterol ester. 1, Cholesterol palmitate; 2, cholesterol oleate; 3, cholesterol linolate; 4, cholesterol linoleate. Separation was performed on HPTLC Kieselgel 60 F_{254} (Merck) in carbon tetrachloride solvent system. (Reproduced with permission from Kovács *et al.*, 1989. Copyright 1989 American Association for the Advancement of Science.)

HPTLC silica gel plates and a dual solvent system, consisting of a run with isopropyl ether–acetic acid (96 : 4) followed by a run in the same direction with petroleum ether (b.p. 60–70°C)–diethyl ether–acetic acid (90 : 10 : 1) has been to determine cholesterol in egg yolk as well as in butter and cream samples. Cholesterol was detected with cupric acetate reagent, lipid zones were quantified by densitometry.

Quantitative measurement of free cholesterol in serum on a silica gel/sodium carboxymethylcellulose plate has also been reported. The solvent system was petroleum ether–ethyl acetate–glacial acetic acid (80 : 20 : 1). Spraying with vanillin was used for visualization. The colour of the cholesterol spot was stable for *c.* 20 min. *In situ* measurement was done by densitometry at 525 nm with a detection limit of 40 ng per spot. The peak area was linearly related to the amount of cholesterol over the range 80–700 ng per spot.

Cholesterol, cholesteryl esters and other neutral lipids have been analysed in plasma by TLC-FID. Separation was performed on Chromarods S with hexane–diethyl ether–formic acid (52 : 8 : 0.1).

Quantitative *in situ* analysis of vitamin D in cod liver oil has been demonstrated by measuring absorbance at 268 nm on silica gel layers after dual development (first with *n*-hexane, then with cyclohexane–diethyl ether (1 : 1) mixture). Vitamin D was also determined in foods and in human milk by *in situ* reflectance measurement.

TLC and Characterization of Sterols

The chromatographic behaviour of each compound depends on the stereochemistry and location of polar

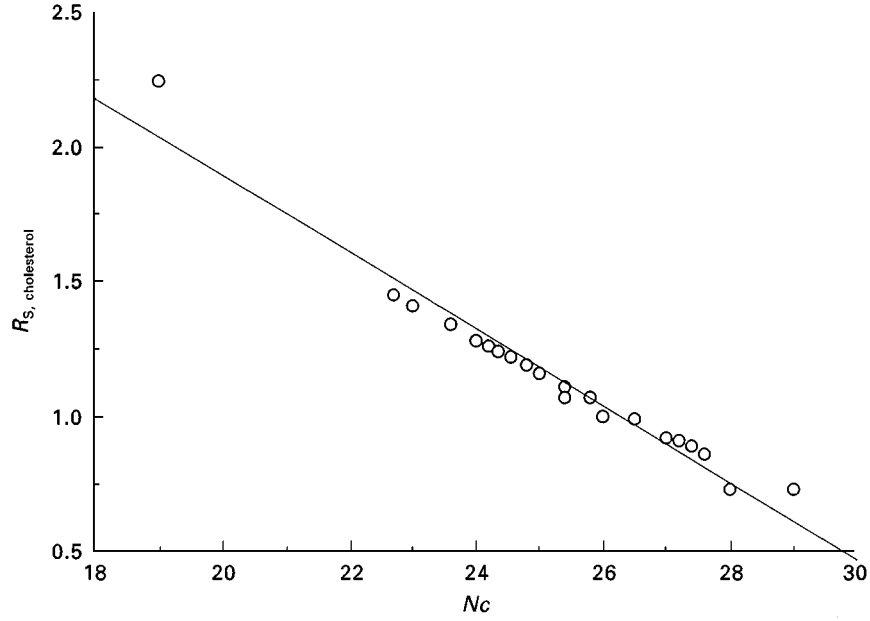

Figure 3 Relationship between R_S values and the carbon numbers for sterol acetates in RP-TLC; coefficient of linear correlation, $R = 0.9793$. Stationary phase: Kieselguhr G impregnated with undecane; mobile phase: acetic acid–acetonitrile (1 : 3). Nc = number of carbon atoms − number of double bonds. (Data from Copius-Peerboom and Beekes, 1965.)

substituents, the solubility, partition coefficients and equilibrium constants of the compound in the solvent system, the size and shape of the molecule and the degree of hydration. The quantitative structure–chromatographic retention relationship study between sterols, TLC mobilities and their structures has been investigated by several authors.

A separation of steroids according to the degree of unsaturation has been investigated. In structural analysis, argentation TLC and the bromine system can give information about the number and position of double bonds in a molecule. A linear relationship between the R_S value and carbon numbers (Nc = number of carbon atoms − number of double bonds) in the system undecane/acetic acid–acetonitrile (1 : 3) for saturated and Δ^5-unsaturated sterols has been found. This linear relationship is shown in **Figure 3**.

Adsorption TLC is not only a method of sample purification, but the R_F value also provides a clue to the compound's structure. The structural feature that mostly contributes to the chromatographic behaviour of sterols in adsorption TLC is the presence of a free 3β-OH group. Converting the 3β-OH to an acetoxy, methoxy, keto, or 3α-OH results in a steroid with a less polar R_F value relative to the R_F value obtained for cholesterol.

Separation of individual cholesterol ester subfractions according to the sum of the carbon atoms and numbers of double bonds in their fatty acid moieties has been performed.

The elution order of vitamin D photoisomers can be correlated with the increasing planarity of the molecules. R_F values of vitamin D photoisomers on silica gel with solvent system cyclohexane–dichlorethane–diethyl ether (5 : 3 : 2) were 0.18 (provitamin D_3), 0.23 (tachysterol$_3$), 0.27 (lumisterol$_3$) and 0.31 (pre-vitamin D_3).

A visualization procedure can give additional information about sterol structure, since different reagents produce different colours with individual sterols. Some reagents are specific for individual functional group (e.g. 2,4-dinitrophenylhydrazine for keto group).

Future Developments

A general tendency in modern TLC is separation on HPTLC stationary phases, online coupling with other separation techniques (e.g. HPLC-TLC), as well as online coupling with spectroscopic methods. *In situ* recording of UV-visible spectra is most commonly used. However, the recording of Fourier transform infrared, Raman or mass spectra is more informative. Although these combinations have frequently been reported in the literature, there is still no example of their application in the field of sterol analysis. Due to the great variety of chemically closely related sterols, online combination of TLC and spectroscopic methods can be considered a powerful tool for their isolation and *in situ* characterization.

See also: **II/Chromatography: Thin-Layer (Planar):** Densitometry and Image Analysis; Spray Reagents. **III/Flame Ionization Detection: Thin-Layer (Planar) Chromatography. Impregnation Techniques: Thin-Layer (Planar) Chromatography. Silver Ion:** Thin-Layer (Planar) Chromatography. **Sterols:** Supercritical Fluid Chromatography.

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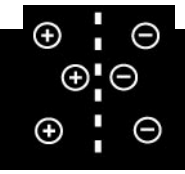
STRONTIUM FROM NUCLEAR WASTES: ION EXCHANGE



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Introduction

The development of new inorganic ion exchange materials for the selective removal of strontium and other radionuclides from nuclear waste has progressed rapidly in recent years. ^{90}Sr is an important component of many nuclear wastes and is a high yield fission product of ^{235}U. It is relatively short-lived with a half-life of 28.8 years and, along with ^{137}Cs, is the source of a large percentage of the initial radioactivity and heat generation of spent nuclear fuel. During the reprocessing of nuclear fuel, irradiated uranium fuel rods are dissolved in nitric acid and uranium and plutonium are separated from the fission products and other actinides by means of the Purex process. Tributylphosphate (TBP) dissolved in an organic phase, such as odourless kerosene, is contacted with the nitric acid solution, and plutonium and uranium nitrates are selectively complexed by the TBP and extracted into the organic phase. The majority of fission products, including ^{90}Sr, remain in the aqueous acidic phase, which can then be concentrated by means of evaporation and stored prior to permanent disposal. In addition to the acidic high level waste stream, numerous other streams are generated during reprocessing operations as a result of washing, decontamination and scrubbing operations. Details of some specific streams generated by the nuclear industry from which ^{90}Sr needs to be selectively removed from large excesses of inert ions will be given later in this article.

A convenient method of selectively removing contaminant species from higher concentrations of inert ions is by ion exchange. Organic ion exchange resins are used in many industries for the selective removal of ions from aqueous streams. These materials consist of a polymeric backbone (commonly polystyrene) to which has been attached functional groups such as carboxylic or sulfonic acids to produce cation exchangers, or tertiary or quartenary amines to produce anion exchange resins. However, the use of organicresins in the nuclear industry is limited for a number of reasons. These include: